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Assessment of plasma acylcarnitines before and after weight loss in obese subjects.

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Abstract

Acylcarnitines, fatty acid oxidation (FAO) intermediates, have been implicated in diet-induced insulin resistance and type 2 diabetes mellitus. Moreover plasma acylcarnitines have been associated with clinical parameters related to glucose metabolism, such as fasting glucose levels and HbA1c. We investigated plasma acylcarnitines in relation to energy metabolism (including energy expenditure (EE)) and insulin sensitivity measurements in 60 obese subjects before and after a 12 week weight loss intervention. Since the acylcarnitine profile reflects FAO at the mitochondrial level, we expected plasma acylcarnitines to correlate with energy expenditure, insulin sensitivity and other clinical parameters before and during a weight loss intervention. In contrast, despite the amelioration of HOMA-IR, plasma acylcarnitines levels increased during weight loss. This is most probably attributable to higher lipolysis and lipid oxidation rates due to the caloric deficit and not by mitochondrial overload and incomplete FAO, as insulin signalling was not impaired. HOMA-IR, energy expenditure and respiratory exchange ratio were not related to plasma acylcarnitines. However a strong correlation between NEFA and several acylcarnitines was found at baseline and during the weight loss intervention, again potentially reflecting lipolysis. Our data show that acylcarnitines do not correlate with clinical parameters of glucose metabolism during weight loss, questioning the role of acylcarnitines in the etiology of insulin resistance and subsequent type 2 diabetes mellitus.

Introduction

With the increased incidence of obesity and type 2 diabetes mellitus, many studies focus on the interaction between lipid and glucose metabolism, and the relationship to insulin resistance. The concept of lipotoxicity has been proposed as a mechanism by which increased lipid levels interfere with insulin signalling and eventually lead to hyperglycemia. However, the exact mechanisms and the individual lipids that induce insulin resistance have not been characterised definitively. From a cellular point of view, lipotoxicity is thought to occur on a cytosolic level via lipid overload (e.g. increased levels of ceramides, gangliosides or diacylglycerol) (1-4). Reduced mitochondrial content or capacity may result in elevated intracellular lipids (3, 5). Alternatively, increased fatty acid oxidation (FAO) rates that are not followed by an increase in tricarboxylic acid cycle (TCA) activity have been proposed to induce insulin resistance via accumulation of different mitochondrial metabolites such as acylcarnitines (1, 6, 7). This incomplete FAO is suggested to be manifested by altered plasma acylcarnitine profiles, mainly increased levels of long chain acylcarnitines (1, 7, 8).

Acylcarnitines are fatty acids esterified to carnitine. They are synthetized by the outer membrane enzyme carnitine palmitoyl tranferase 1 (CPT1) to enable transmitochondrial membrane transport of long-chain acyl-CoAs. Inside the mitochondrion, carnitine is exchanged for CoA via the inner mitochondrial membrane enzyme CPT2. Here the released acyl-CoA can be further oxidized via beta-oxidation, in which the acyl-CoA is shortened by one acetyl-CoA unit in every round. CPT-2 can convert acyl-CoAs into acylcarnitines again, which can be shuttled back into the cytosol and exported to the plasma compartment, ultimately contributing to the typical circulating profile of acylcarnitines (9). Consequently, acylcarnitines are excellent indicators of altered FAO as demonstrated by conditions in which lipid oxidation rates are elevated or when lipid oxidation is impaired (e.g. short-term fasting and FAO disorders). More recently, metabolomic studies have shown that acylcarnitines may be implicated in insulin resistance (7, 8, 10), as elevated acylcarnitine levels are found in both rodent models of dietary insulin resistance (7) and in obese, insulin resistant humans (8). Also several acylcarnitine species

correlate moderately with clinical markers such as BMI, plasma glucose levels and insulin sensitivity in humans with obesity and type 2 diabetes mellitus (8, 10).

Here we report on plasma acylcarnitine concentrations before and during weight loss in obese human subjects. Based on the association of long- and short-chain acylcarnitines with type 2 diabetes mellitus, we hypothesized that these acylcarnitine levels would decrease with concomitant improvements in insulin sensitivity. In contrast, we observed that decreased body weight and improvements in insulin sensitivity were accompanied by increased plasma acylcarnitine levels. Moreover, we found that plasma acylcarnitines correlate strongly with plasma non-esterified fatty acids (NEFA).

Research design and methods

Design of the study

Sixty obese subjects were recruited to take part in an outpatient study for weight loss prediction that has been reported elsewhere (11). In brief, subjects aged 20-55 years and BMI 30-40 kg/m^2 were included. Exclusion criteria were as follows: type 2 diabetes mellitus, history of childhood obesity and previous bariatric surgery. After giving informed consent, subjects were randomized to one of three 12-week weight loss interventions: (1) diet (-600 kcal/day) alone, (2) the same diet with moderate exercise (~10% of daily expenditure), and (3) the same diet with the centrally acting serotonin-norepinephrine reuptake inhibitor, sibutramine, which was approved for weight loss at the time this study was conducted. During the study, subjects visited the clinical unit at 0, 4 and 12 weeks at 07:00 hours a.m. after an overnight fast for the measurement of body weight, anthropometry, indirect calorimetry and blood sampling (e.g. plasma acylcarnitine, glucose, non-esterified fatty acids and insulin levels). The study was approved by the protocol review panel of GlaxoSmithKline, the Cambridge Local Research Ethics Committee (08/H0308/10) and the Wellcome Trust Clinical Research Facility Scientific Advisory Board. Subjects gave written informed consent before participation.

Body weight measurement and anthropometry by DXA

Body weight was measured in light clothing. Body composition was analysed by DXA (GE Lunar Prodigy, software version 12.2 (GE Healthcare, Madison, WI) and quantitative magnetic resonance (Echo MRI-AH; Echo Medical Systems, Houston TX). Indirect calorimetry was performed using a ventilated canopy calorimetry instrument (GEM Nutrition, Daresbury, UK) with the subject lying supine for 20 minutes before the measurement. Expired gas samples were analyzed every 30 seconds for 20 minutes. Gas exchanges of O_2 and CO_2 were computed to calculate respiratory exchange ratio (RER) and energy expenditure (EE; kJ/min using the following formula: 15.9131 x O_2 consumption + 5.2069 x CO_2 production x 0.9950) (12).

Laboratory analyses

For plasma acylcarnitine analysis, 50 µl of plasma was mixed with 100 µl of internal standard mixture (50 µl of 5 µM $^{[3,3,3\text{-}2H3]}$C3-carnitine and 2 µM $^{[6,6,6\text{-}2H3]}$C6-, $^{[8,8,8\text{-}2H3]}$C8-, $^{[10,10,10\text{-}2H3]}$C10- and $^{[16,16,16\text{-}2H3]}$C16-carnitine in acetonitrile (ACN), and 50 µL of 26 µM [methyl-2H3]-L-carnitine in 10% ACN) [38]. The plasma samples were deproteinized by addition of 500 µL ACN followed by vortex mixing. Next, samples were centrifuged for 10 minutes at 4°C at 20.000 *g*. The supernatant was transferred into 4 mL glass vials and evaporated under a stream of nitrogen at 40°C. After evaporation, 100 µL butylation reagent (4:1 mixture of 1-butanol and acetylchloride) was added and incubated for 15 minutes at 60°C. Again evaporation was performed at 40°C. The residue was dissolved in 100 µL ACN, vortex mixed and transferred to Gilson vials for tandem mass spectrometric analysis (Waters/Micromass Quattro Premier XE). Acylcarnitine measurements were processed with Masslynx software version 4.1.

Acylcarnitines are depicted as C followed by chain length and degree of saturation. The acylcarnitines which we analysed were as follows: Free carnitine (C0), acetylcarnitine (C2; derived from both lipid and carbohydrate oxidation (CHO)), hydroxybutyrylcarnitine (C4OH; the sum of the *L* and *D* stereoisomers derived from FAO and ketone bodies respectively (13)),

decanoylcarnitine and tetradecenoylcarnitine (C10 and C14:1 respectively; intermediates that are only produced by FAO and thus indicative of FAO rate) and finally palmitoylcarnitine and oleoylcarnitine (C16 and C18:1 respectively; intermediates that originate from the diet).

Glucose was measured using a hexokinase assay. Insulin was measured using a fluorometric autoDELFIA immunoassay. Plasma free fatty acids were analysed with an NEFA-HR(2) in vitro enzymatic colorimetric method (Wako Diagnostics, Richmond VA).

Statistical analysis

For differences between subgroups at baseline, one-way ANOVA with Bonferroni correction was performed. To determine if plasma acylcarnitine levels at baseline predicted clinical parameters, Pearson correlation analyses were performed followed by Bonferroni correction. In case of significant results, multiple regression analysis was done to establish which acylcarnitine had the greatest effect on a single clinical parameter. Differences between days 0, 28 and 84 for whole group and within subgroup data were analysed using repetitive ANOVA analysis with Bonferroni correction. Statistical analysis was done with SPSS statistical software program version 20.0. Data are depicted as mean and standard deviation.

To analyse if changes in plasma acylcarnitine levels over time coincided with changes in clinical parameters, we used a Bayesian hierarchical model with fixed and random effects. Individual variables were modelled by linear regression over time. For instance, changes in weight were modelled as: $W_i(t) = \alpha_i^{\omega} + \beta_i^{\omega} t + \varepsilon_{it}^{\omega}$. Here $W_i(t)$ is the weight of subject *i* at time *t*, α_i^{ω} is the mean weight of subject *i*, $\beta_i^{\omega} t$ is the rate of change of the subject's weight over time *t*, and $\varepsilon_{it}^{\omega}$ is a zero mean Gaussian error term: $\varepsilon_{it}^{\omega} \sim N(0, \sigma^2)$. Similarly, each of the acylcarnitines of interest was modelled for each individual patient by linear regression ($C_i(t) = \alpha_i^{c} + \beta_i^{c} t + \varepsilon_{it}^{c}$, where C is any acylcarnitine of interest). To analyse the correlation over time between individual clinical parameters (for example, weight) and acylcarnitines, we modeled β_i^{ω} as a linear regression over β_i^{c} : $\beta_i^{\omega} = \alpha + \beta\beta_i^{c} + \varepsilon_i$ where ε_i is again a zero mean Gaussian error term. We then applied Gibbs sampling to simulate from the posterior distribution using standard software (Just

Another Gibbs Sampler (JAGS, version 3.4.0, http://mcmc-jags.sourceforge.net/)). We used a single Markov chain with a burn-in of 100000 sweeps and then output the values α and β for a further 1000000 sweeps. The proportion of sweeps when β is greater than zero is then the posterior probability that variables are positively correlated, and the proportion of sweeps when $\beta < 0$ is the posterior probability that variables are negatively correlated. This procedure was repeated for all clinical parameters and acylcarnitines of interest followed by the Bonferroni-Holm method to allow for multiple testing (14).

Results

Whole group clinical data and plasma acylcarnitines at baseline (day 0)

Table 1 shows the clinical characteristics of the subjects at day 0 of the study. More women were included then men, and a substantial part of the subjects showed slight impaired fasting plasma glucose levels (FPG) (15), as well as an elevated homeostatic model assessment of insulin resistance (HOMA-IR) index (16). Table 1 additionally shows the levels of the plasma acylcarnitine, of which plasma C16- and C18:1-carnitine levels of our obese subjects were below clinical laboratory reference values. The levels of other acylcarnitines detected within the spectrum are given in the online supplemental data (Supplemental table 1).

To detect relationships between plasma acylcarnitines and clinical parameters at baseline, we performed Spearman correlation analyses. Our analysis showed that plasma NEFA correlated strongly with C2, C4OH, C14:1, C16 and C18:1-carnitine (Table 2 and figure 1). No other correlations were found (Supplemental table 2). Multiple regression identified C14:1 and C16-carnitine as contributors to the variation in plasma NEFA (Table 3). When C4OH-carnitine was omitted from the regression analysis (since this is an acylcarnitine that emerges predominantly during starvation (13)), only C16-carnitine remained significant.

Whole group clinical data and plasma acylcarnitines during the weight loss intervention

Figure 2 shows the effects of the weight loss interventions for the whole group, irrespective of the randomized treatment allocation. Overall, weight decreased significantly between days 0 and 84 (~4.5 kg). As a consequence, BMI decreased between 0 and 28 days, with no additional significant reduction between days 28 and 84. HOMA-IR, FPG and plasma insulin levels improved significantly between days 0 and 28, with no further improvement hereafter (figure 2). Plasma NEFA levels were unaffected by the intervention. Body composition analysis revealed that fat mass continued to decrease during the entire weight loss intervention period although this effect was most pronounced during the first 28 days. Lean body mass decreased significantly (~0.6 kg) until 28 days only (figure 2). Substrate oxidation measurements by indirect calorimetry showed that both EE and RER were significantly lower after 28 days of treatment, with no further reduction afterwards (figure 2). FAO rates did not change upon weight loss. However protein oxidation rates and CHO rates both significantly decreased after 28 days and remained at this level at 84 days (Supplemental figure 1). The decrease in protein oxidation was minor, therefore the decrease in energy expenditure mainly resulted from lower carbohydrate oxidation. Overall clinical improvements due to the weight loss intervention were greatest in the first 28 days, with no further improvement at 84 days.

Whole group plasma acylcarnitine levels for days 0, 28 and 84 are shown in figure 3. The weight loss intervention resulted in an increase in free carnitine levels after 84 days. C2-carnitine and C4OH-carnitine were higher after 28 days with no further increase at 84 days. C14:1- and C18:1-carnitine showed a similar pattern over time with an initial increase in plasma levels after 28 days, followed by a decrease at 84 days, although at this point these acylcarnitine levels were still higher compared with day 0. C16-carnitine was significantly higher after 28 days followed again by a decrease to baseline levels (figure 3).

We used a Bayesian hierarchical model to investigate whether changes in plasma acylcarnitine levels over time correlated with changes in weight, fat mass, lean mass, HOMA-IR, FPG, NEFA, RER and EE. We found that the increase over time in C4OH-, C16- and C18:1-carnitine correlated significantly with a reduction in both total and lean body weight over time (Supplemental table 2

and supplemental figure 2). Additionally over time NEFA levels correlated positively with the level of C16-carnitine (Supplemental table 2 and supplemental figure 2). We found no significant correlations for the other clinical parameters (data not shown).

Subgroup clinical data and plasma acylcarnitines at baseline (day 0)

Table 1 shows the demographic data at baseline for the subgroups. Although in general there were no differences between the groups, there were a few exceptions that were primarily driven by the exercise and sibutramine group. Plasma glucose and RER were both higher in the sibutramine group when compared to the exercise group. A difference in RER was also found for FAO and CHO rates (Table 1). Plasma NEFA levels were lower in the exercise group when compared to the other two groups (Table 1).

Subgroup clinical data and plasma acylcarnitines during the weight loss intervention

The sibutramine group showed continued weight loss up to day 84 in contrast to the other two groups that only showed significant weight loss at day 28, but then remained weight stable up to day 84. The sibutramine group had a significant reduction in HOMA-IR at day 28, after which the levels plateaued in contrast to the exercise and placebo groups where HOMA-IR did not change during the study. Plasma NEFA did not change in any of the three groups. In the exercise and placebo groups, RER was not affected by the weight loss intervention, but the sibutramine group had a lower RER after 28 days that remained stable thereafter.

Plasma acylcarnitine levels showed differential changes during the weight loss intervention. Overall the greatest effect was seen in the sibutramine group and a modest effect was seen for the exercise group. In the placebo group, C0-carnitine did not change, whereas the exercise group showed a modest increase at day 84 compared to day 28. In the sibutramine group, C0-carnitine increased steadily during the intervention becoming significant at day 84. In contrast to the placebo and exercise group, C2- and C4OH-carnitine levels in the sibutramine group showed an increase at 28 days and remained high at 84 days. In the placebo and sibutramine

group, C10- and C14:1-carnitine initially increased at day 28 but not in the exercise group. C16- and C18:1-carnitine were higher at day 28 compared to day 0 in all groups, with the exception of C16-carnitine in the exercise group. At day 84, C10- and C18:1-carnitine were significantly decreased again in the placebo group compared to day 28.

We repeated the Bayesian hierarchical modeling to determine if changes in plasma acylcarnitine levels overtime correlated with changes in clinical parameters and found that the change in plasma C4OH- and C18:1-carnitine remained significantly correlated with weight change in the sibutramine group, but not in the other groups (data not shown).

Discussion

It has been suggested that acylcarnitines play a role in insulin resistance (1, 17). In this study we have shown in obese humans that upon weight loss the improvement in insulin sensitivity was accompanied by a significant increase in acylcarnitine levels. Additionally the plasma levels of acylcarnitines increased and acylcarnitines correlated positively with NEFA at baseline and over time.

Several studies have reported that increased plasma acylcarnitine levels associate with obesity and insulin resistance (7, 8, 10, 18). Adams et al reported that C2-carnitine, derived from both lipid and carbohydrate oxidation, correlated positively with HbA1c in diabetic subjects (10). Mihalik et al showed multiple correlations of short and longer chain acylcarnitines with glucose metabolism (8). Although they did not report C2-carnitine values, they found strong correlations with C4-dicarboxylcarnitine (C4-DC-CN) with fasting plasma glucose levels and HbA1c. In our study, plasma NEFA correlated positively with plasma C16-carnitine and other species, namely C2-, C14:1- and C18:1-carnitine. With respect to this strong correlation of acylcarnitines with plasma NEFA, which are known to induce insulin resistance, the overall absence of correlations between acylcarnitines and markers of insulin sensitivity is remarkable (19, 20). Since plasma NEFA are indicative of lipolysis, acylcarnitines in the plasma may reflect white adipose tissue (WAT) breakdown (21). As a result, the NEFA released from WAT could drive FAO rates generating acylcarnitines.

The origin of the different acylcarnitines that correlate with plasma NEFA is intriguing as well. As discussed above, C2-carnitine can be derived from both lipid and carbohydrate oxidation and our observation of a correlation between C2-carnitine and plasma NEFA suggests that lipid is the main source in our subjects (22, 23). C16- and C18:1-carnitine are derived from palmitate and oleate, which are the main human dietary fatty acids. Their correlation with plasma NEFA may support lipolysis as a responsible mechanism, since these dietary fatty acids are stored in WAT. Finally, C14:1-carnitine is an interesting acylcarnitine since it is only produced after two cycles of beta-oxidation of C18:1-CoA (24). Therefore C14:1-carnitine is a good marker of FAO. It

remains unclear what the tissue origin of this acylcarnitine is, but as FAO also takes place in WAT, C14:1-carnitine could still be derived from WAT (25, 26). Alternatively, plasma NEFA may reflect the load of fatty acids in general, thereby correlating with the most metabolically relevant acylcarnitines.

Following these observations at baseline, we studied the effects of a weight loss intervention on acylcarnitines and insulin resistance. Effects of a weight loss intervention on acylcarnitine profiles have been described in only two studies, both of which studied lean subjects (27, 28). Redman et al compared caloric restriction with and without exercise in non-obese men and women, and showed no changes in acylcarnitines in the former group, but increased acylcarnitines in the latter, accompanied by a greater improvement in insulin sensitivity analogous to our study (28). Here, caloric restriction combined with exercise possibly improves the coupling of FAO and TCA flux, preventing acylcarnitines from accumulating. Falk-Petersen et al demonstrated in lean sedentary insulin resistant offspring of parents with type 2 diabetes (27) that plasma acylcarnitine levels do not change after a 9 week hypocaloric diet, despite weight reduction and improved insulin sensitivity. In this case, the unchanged plasma acylcarnitines contrast with our results. These subjects were different from ours with respect to both body composition and insulin sensitivity. Whether differences in mitochondrial lipid flux handling or lipolysis explain the differential effects of the diet on plasma acylcarnitines remains elusive. Also, in these particular subjects, the initial insulin resistance may have been a result of increased intramyocellular lipids (IMCL) rather than acylcarnitines. Finally, the genetic susceptibility of insulin resistant offspring potentially blurs the etiological mechanism of insulin resistance (29). For both studies, it should be emphasized that plasma acylcarnitines do not reliably reflect individual tissue metabolite levels as we have shown recently (26, 30).

Although we did not find a convincing correlation at baseline between plasma acylcarnitine levels and HOMA-IR, changes over time show that the clear improvement in insulin sensitivity was accompanied by a significant increase in acylcarnitine levels. Despite this improvement in insulin sensitivity, carbohydrate oxidation rates decreased, probably reflecting the hypocaloric

state which causes an increase in FAO. Additionally the diet-induced lipolysis generates NEFA that can directly inhibit carbohydrate oxidation as well.

The changes in individual chain lengths of acylcarnitines are important because they reveal some insight on physiological mechanisms in acylcarnitine metabolism during weight loss. Carnitine availability depends on dietary intake and endogenous synthesis. Both carnitine uptake via OCTN2 and carnitine synthesis are regulated by PPAR-alpha and are stimulated under hypocaloric conditions (17). The absence of increased free carnitine after 28 days of intervention may reflect increased esterification to fatty acids and efflux of toxic lipid metabolites. This effect was probably lessened after 84 days when weight loss declined and free carnitine levels increased. Indeed, the increase in lipid derived acylcarnitines, including C2-carnitine, was most pronounced after the first 4 weeks. Here the hypocaloric condition and subsequent weight loss could cause an improvement of CrAT activity with increased production of C2-carnitine, and long chain acylcarnitines that are released from the mitochondria and into the plasma compartment (31). When this diet induced CrAT activity declines again over time, more free carnitine is released which explains the delay in this increase of free carnitine levels. These changes over time were not seen for the amino acid derived acylcarnitines, which shows that under hypocaloric conditions lean body mass is more protected than fat mass (32).

Overall, acylcarnitines increased and insulin sensitivity improved in our study and so our results dissociate insulin sensitivity from plasma acylcarnitines. We speculate that the elevated acylcarnitine levels on day 28 and day 84 may reflect lipolysis, due to the caloric deficit of the weight loss interventions as detailed above. Additionally it could reflect higher rates of FAO and CRAT activity, although we could not confirm this with measurements of whole body lipid oxidation or acylcarnitines in tissue.

The effects on both acylcarnitine levels and the clinical parameters, such as weight loss, were greatest at 28 days and were weaker at 84 days. It is often seen in weight loss studies that the initial effects outweigh the later effects, which may not only be due to changes in treatment compliance over time (33-35). Under hypocaloric conditions, whole body energy expenditure

decreases hampering further weight loss once a lower body weight is attained (36-38). The increase in acylcarnitines over time also showed a relation with weight loss. Here, a stronger decrease in total and lean body weight correlated with higher C4-OH-, C16- and C18:1-acylcarnitine levels.

Although we have analysed and reported on whole group changes over time because of the limited number of subjects, the weight loss intervention consisted of three arms (diet, sibutramine and exercise) (11). We performed mainly within-group analyses because of the primary interest in changes in acylcarnitines over time. However, the different interventions are intriguing and reveal subtle, but clear effects on clinical parameters, and acylcarnitine metabolism in particular. Over time the sibutramine group lost the most weight and had the highest acylcarnitine levels, in contrast to more modest effects in the other two groups. This may confirm the idea that lipolysis, which is apparently greater when weight loss is greater, is reflected as higher levels of plasma acylcarnitines. Weight loss in the other two groups was comparable, but the rise in acylcarnitines was nearly absent upon weight loss in the exercise group. This may be due to more efficient mitochondrial fatty acid oxidation due to exercise (28, 39, 40), which could reduce the accumulation of acylcarnitines in the plasma compartment. Although the different interventions were extremely instructive for our understanding of acylcarnitine metabolism, the subgroups do have limitations, as the groups were relatively small, the exercise intervention was only modest and the marketing application for sibutramine has been withdrawn due to side effects of the drug (41).

In conclusion, we have found an increase in several acylcarnitine species in association with weight loss in obese human subjects, despite improvements in insulin sensitivity. It is likely that the level of acylcarnitines in plasma is driven by the rate of lipolysis as well as improved efflux from cells, potentially as a result of improved CrAT activity and not by deranged mitochondrial FAO. The diet derived acylcarnitines, C16- and C18:1-carnitine, seem the most relevant acylcarnitines at baseline and during weight loss. However, the importance of plasma

acylcarnitines as clinical markers for insulin resistance seems negligible and the interpretation of changes in these lipid intermediates in relation to clinical status remains challenging.

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AUTHOR CONTRIBUTIONS M.G.S. performed plasma acylcarnitine analyses, analysed data and wrote the paper. A.N. designed and performed the research, analysed data and wrote the paper. S.M.H. analysed data and wrote the paper. G.K.A. performed statistical analyses and wrote the paper. P.R.M. designed the research and analysed data. S.R.M. designed the research, analysed data and wrote the paper. C.Y.T. performed the research, analysed data and wrote the paper. S.V. wrote the paper. A.V.-P. designed the research and wrote the paper. D.J.N. designed the research and wrote the paper. M.R.S. designed the research, analysed data and wrote the paper.

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Table 1 *Baseline characteristics, clinical parameters and plasma acylcarnitine levels*

	Total	Diet	Exercise	Sibutramine	
Subjects (*N)*	60	20	21	19	
Age (years)	40 (± 8.6)	41 (± 7)	41 (± 8)	40 (± 11)	
Sex (*m/f*)	23/37	10/10	7/14	6/13	
Bodyweight (kg)	100.9 (± 12.6)	105.0 (± 12.7)	98.7 (± 11.7)	99.2 (± 13.0)	
BMI (kg/m2)	34.8 (± 2.7)	35.2 (± 2.2)	34.5 (± 3.0)	34.8 (± 2.8)	
Fat mass (kg)	43.8 (± 7.6)	43.0 (± 9.1)	41.4 (± 11.5)	43.2 (± 8.4)	
Lean mass (kg)	52.2 (±10.9)	56.4 (± 11.7)	52.2 (± 10.9)	51.0 (± 9.7)	
EE (kJ/min)	5.2 (± 0.8)	5.4 (± 0.7)	5.2 (± 0.9)	5.1 (± 0.8)	
RER	0.81 (± 0.05)	0.81 (± 0.04)	0.79 (± 0.04)	0.84 (± 0.06)*a*	
FPG (mmol/L)	5.6 (± 0.5)	5.8 (± 0.5)	5.4 (± 0.3)	5.7 (± 0.6)*a*	
Insulin (pmol/L)	14.7 (± 10.1)	13.2 (± 11.3)	13.2 (± 6.2)	17.9 (± 11.9)	
HOMA-IR	3.7 (± 2.9)	3.5 (± 3.4)	3.2 (± 1.6)	4.6 (± 3.3)	
NEFA (mmol/L)	0.5163 (±0.18)	0.49 (± 0.16)*b*	0.50 (± 0.18)	0.56 (± 0.19)*a*	
Acylcarnitines					*Ref values*
C0 (µmol/L)	32.9 (± 7.8)	33.5 (± 7.6)	34.3 (± 6.8)	31 (± 9.1)	*22.30 – 54.80*
C2 (µmol/L)	4.86 (± 1.68)	4.55 (± 0.96)	5.06 (± 1.13)	4.96 (± 2.56)	*3.40 – 13.00*
C4OH (µmol/L)	0.026 (± 0.021)	0.02 (± 0.012)	0.027 (± 0.012)	0.03 (± 0.031)	*0.00 – 0.15*
C10 (µmol/L)	0.18 (±0.11)	0.16 (± 0.10)	0.020 (± 0.13)	0.18 (± 0.08)	*0.04 – 0.30*
C14:1 (µmol/L)	0.079 (±0.035)	0.07 (± 0.03)	0.085 (± 0.036)	0.08 (± 0.036)	*0.02 – 0.18*
C16 (µmol/L)	0.024 (± 0.012)	0.021 (± 0.008)	0.026 (± 0.014)	0.024 (± 0.011)	*0.06 – 0.24*
C18:1 (µmol/L)	0.028 (±0.009)	0.029 (± 0.008)	0.028 (± 0.009)	0.028 (± 0.012)	*0.06 – 0.28*

BMI, body mass index; EE, energy expenditure; RER, respiratory exchange ratio; FPG, fasting plasma glucose; HOMA-IR, homeostatic model assessment of insulin resistance; NEFA, non-esterified fatty acids. Acylcarnitine reference values were acquired and validated by Laboratory Genetic Metabolic Diseases, Academic Medical Center, Amsterdam. Data are represented as mean ± standard deviation; *a* = statistical significant between exercise and sibutramine, *b* = significant between exercise and placebo.

Table 2 *Correlations of plasma NEFA with plasma acylcarnitines*

	Plasma NEFA	
	Spearman **ρ**	p
C0	0.24	0.07
C2	0.50	0.00 *
C4OH	0.40	0.00 *
C10	0.22	0.11
C14:1	0.53	0.00 *
C16	0.60	0.00 *
C18:1	0.46	0.00 *

Spearman correlation analysis shows a strong correlation of C2-, C4OH-, C14:1-, C16- and C18:1-carnitine with plasma NEFA at baseline. P-values are shown before Bonferroni correction whereas * refers to p-values that remained significant after Bonferroni correction.

Table 3 *Multiple regression analysis of plasma NEFA levels and selected plasma acylcarnitines*

Analysis including C4OH-carnitine			Analysis excluding C4OH-carnitine		
	Unstandardized Coefficient B	*p*		Unstandardized Coefficient B	*p*
C0	-0.01	0.19	C0	-0.00	0.54
C2	0.05	0.12	C2	0.01	0.72
C4OH	-3.40	0.08			
C10	-0.39	0.16	C10	-0.25	0.36
C14:1	2.55	0.04 *	C14:1	1.84	0.11
C16	2.51	0.04 *	C16	3.07	0.01 *
C18:1	-0.13	0.91	C18:1	-0.16	0.89

Multiple regression analysis illustrates that variation in plasma NEFA is driven by C14:1 and C16-carnitine. When C4OH-carnitine was omitted (as it predominantly emerges during starvation), only C16-carnitine remained significant.